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Lipopolysaccharide quantification and alkali-based inactivation in polysaccharide preparations to enable *in vitro* immune modulatory studies



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ABSTRACT

The correct identification of immune-modulatory activity of polysaccharides is often hampered by immune-stimulatory contaminants, with pyrogens such as lipopolysaccharide (LPS) as a very potent example. In order to avoid false positive immuno-stimulatory properties to be attributed to polysaccharides, accurate quantification and inactivation of LPS in test samples is crucial. To quantify LPS in polysaccharide preparations of different origin and structure we used two different limulus amoebocyte lysate test kits in two different laboratories. We observed larger variation in detection of LPS contamination between kits than between labs. LPS quantification proved unreliable for some polysaccharide preparations as spike controls resulted in spike recoveries outside the acceptable range. We designed a cellular *in vitro* assay as alternative method to detect the presence of functional LPS. This HEK-Blue hTLR4 cell culture provides a reliable assay, when combined with a cell viability test, for determining functional LPS in polysaccharide preparations. Finally, to inactivate LPS in polysaccharide preparations, we setup an alkaline-ethanol-based treatment. With this assay we observed that our treatment (5 h incubation in 0.1 M NaOH) at 56 °C efficiently inactivated LPS in all polysaccharide preparations below immune-stimulatory levels. At this elevated temperature, however, we also observed minimal to severe degradation of polysaccharide preparations as determined with SEC-RH. Taken together, we describe methods and precautions to reliably detect and inactivate LPS in polysaccharide preparations to allow reliable *in vitro* investigations towards immune-modulatory potential of polysaccharide preparations.

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1. Introduction

In recent years, polysaccharides have received increasing interest as potential immune-modulators (Schepetkin & Quinn, 2006; Wismar, Brix, Frokiaer, & Laerke, 2010). Anti-infectious (Estrada et al., 1997; Jung et al., 2004; Murphy et al., 2008; Yun et al., 1998) and anti-tumor (Harada, Itashiki, Takenawa, & Ueyama, 2010; Hong et al., 2004; Modak, Koehne, Vickers, O'Reilly, & Cheung, 2005) activities of several polysaccharides have been demonstrated in animal models. The mechanisms behind these effects are only partially understood, even though a range of polysaccharide receptors has been identified on various immune cells (Wismar et al., 2010). These carbohydrate binding receptors

belong to the group of pattern recognition receptors (PRRs) that include c-type lectins, nucleotide-binding oligomerization domain (NOD)-like receptors, scavenger receptors and toll-like receptors (TLRs) (Mogensen, 2009).

In vitro and *ex vivo* studies investigating interactions between polysaccharides and immune cells can make a significant contribution to improve understanding of the diverse effects of polysaccharides on the immune system. However, the potential presence of immuno-stimulatory contaminants in polysaccharide preparations, such as microbial pyrogens, complicates this task. One of the most potent and ubiquitous immune-stimulatory contaminants is lipopolysaccharide (LPS) or endotoxin. LPS is comprised of three regions: the O-polysaccharide; the core component; and lipid A. The O-polysaccharide comprises the outer domain of LPS and consists of repeating oligosaccharide subunits that can vary greatly between bacterial strains (Lerouge et al., 2001). The core component, consisting of hetero-oligosaccharides, links the O-chain to lipid A. The biological activity of LPS is harboured in

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Lipid A (Mueller et al., 2004), which is also the most structurally conserved region of LPS (Tzeng, Datta, Kolli, Carlson, & Stephens, 2002). It consists of a phosphorylated glucosamine hetero-disaccharide acetylated with 6 fatty acid chains (Lerouge et al., 2001). Immune responses to LPS are initiated via the LPS receptor complex, which follows sub-sequential actions of LPS binding protein (LBP), CD14, lipid binding accessory protein (MD-2) and TLR-4 (Rossol et al., 2011). This complex can induce cytokine secretion by dendritic cells *in vitro* to LPS concentrations as low as 20 pg/ml (Schwarz, Schmittner, Duschl, & Horejs-Hoeck, 2014; Tynan, McNaughton, Jarnicki, Tsuji, & Lavelle, 2012).

There are many examples of false-positive immuno-stimulatory activities of plant extracts or polysaccharide preparations reported in the scientific literature that were later attributed to LPS contamination (Pugh et al., 2008; Rieder et al., 2013; Tamta et al., 2008). To obtain valuable results from *in vitro* and *ex vivo* studies regarding immuno-modulation, adequate endotoxin control is crucial (Gertsch, Viveros-Paredes, & Taylor, 2011). Activation of the clotting cascade of limulus amoebocyte lysate (LAL) from the horseshoe crab (*Limulus polyphemus*) has been the standard assay for LPS quantification for many years due to its high sensitivity (Gutsmann et al., 2010). However, the quantification of LPS is particularly difficult in polysaccharides as most methods have been developed for protein samples. In addition, LAL-based assays have also been reported to give “varying results” for protein preparations (Schwarz et al., 2014). One standard method to block LPS activity *in vitro* is the use of Polymyxin B (PmB), an antibiotic with a high binding affinity for Lipid A (Moore, Bates, & Hancock, 1986). Even though this method is convenient and effective for many samples, it is not universally applicable. In some polysaccharide preparations, PmB-mediated LPS inactivation is ineffective (Rieder et al., 2013). Interestingly, PmB is effective upon degradation of the polysaccharide sample. The ineffectiveness of PmB in certain polysaccharide preparations may therefore be due to the formation of a polysaccharide-LPS complex that hinders binding of LPS to PmB (Rieder et al., 2013). Furthermore, PmB ability to inhibit LPS-induced cytokine secretion from dendritic cells was shown to be incomplete (Tynan et al., 2012). Alternative reliable methods for LPS quantification in polysaccharide samples are therefore needed.

Here, we describe LPS quantification and inactivation in different polysaccharide preparations. We compare the LAL-based EndoZyme and ToxinSensor kits in two different laboratories and demonstrate that these kits are not universally applicable for polysaccharide preparations. As an alternative, we show that HEK-Blue hTLR4 cells can reliably determine functional LPS levels. We next applied an alkaline-ethanol treatment at 4 °C and 56 °C to inactivate LPS in these polysaccharide preparations. At 56 °C we observed LPS inactivation below detection thresholds of the HEK-Blue hTLR4 assay. To monitor possible degradation of the polysaccharides, size exclusion chromatography (SEC-RI) was selected as a screening tool which revealed polysaccharide- and temperature-dependent changes in molar masses.

2. Materials and methods

2.1. Cell culture

HEK-Blue hTLR4 cells (InvivoGen, Toulouse, France) were sub-cultured in DMEM (Gibco, Life Technologies, Bleiswijk, The Netherlands) with 10% fetal bovine serum (FBS; Gibco) once per week and medium was refreshed twice per week.

2.2. Polysaccharide preparations

The polysaccharide preparations employed in this study originate from baker's yeast (*Saccharomyces cerevisiae*), Shiitake

mushroom (*Lentinus edodes*), wheat (*Triticum aestivum*), oat (*Avena sativa*) and apple (*Malus domestica*). Water soluble 1,3/1,6 beta-glucan from yeast (Wellmune Soluble) was provided by Biothera (Eagan, MN, USA) and contained > 90% β -glucan (according to the manufacturer). Linear, mixed linkage β -1,3/1,4-glucan from oat with a purity of > 90% (OBG90) was provided by Swedish Oat Fiber (Bua, Sweden). A water soluble, arabinoxylan-enriched, fraction from wheat (Naxus) (55% arabinoxylan based on sum of arabinose and xylose) was provided by Bioactor (Maastricht, The Netherlands). A rhamnogalacturonan-I fraction from apple pectin (RG-1) (76% pectin based on the sum of rhamnose, arabinose, galactose and uronic acid) was provided by INRA (INRA, Nantes, France). Lentinan containing extract from shiitake (LCES; 51% β -glucan based on starch-free glucose content) was acquired based on a previously described method (Tomassen, Hendrix, Sonnenberg, Wichers, & Mes, 2011). In brief, 660 gr fresh shiitake was homogenized in a magimix blender. The grounded pieces were added to 1 l of boiling tap water. The samples were further homogenized with a blender and boiled for 8 h. After O/N cooling the extracts were centrifuged at 2800 rpm for 30 min. The supernatant was collected and precipitated O/N by addition of 1 vol of 96% ethanol. The precipitate was collected and lyophilized. The constituent sugar content used for purity estimation is based on alditol acetate derivatives quantified by GC after acidic hydrolysis and derivatization (Blakeney, Harris, Henry, & Stone, 1983).

2.3. Preparation of polysaccharide samples for LPS quantification and cell culture experiments

At lab 1, the polysaccharide samples were dissolved in endotoxin free water (< 0.005 EU/ml, G-Biosciences, MO, USA) at a concentration of 1 mg/ml in endotoxin free centrifuge tubes (< 0.05 EU/ml following water washing of tubes) by incubation in a boiling water bath for 1 h. Prior to LPS quantification by the LAL method, samples were diluted stepwise (1:10) with endotoxin free water. At lab 2, the polysaccharide samples were dispersed in endotoxin free water (Life Technologies-Gibco, Bleiswijk, The Netherlands) in endotoxin-free glass vials (Hyglos, Bernried am Stranberger See, Germany) at a concentration of 3 mg/ml and stirred for 6 h and stored overnight (O/N) at ambient temperature (RT). Subsequently, samples were diluted with endotoxin free water to 0.75 or 1 μ g/ml for quantification of LPS by the LAL method. For use in cell culture experiments, polysaccharides were dispersed O/N in appropriate endotoxin free culture medium at 40 °C.

2.4. LPS quantification

2.4.1. LPS quantification with commercial LAL based test kits

LPS quantification in the different polysaccharide samples (prepared as described above) was conducted with two different commercial test kits. Both test kits did not contain the specific recognition protein for β -glucan (factor G). ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit was purchased from GenScript (GenScript, NJ, USA). EndoZyme® test kit, which contains recombinant factor C, was purchased from Hyglos (Hyglos GmbH, Bernried am Stranberger See, Germany). Both test kits were used according to the manufacturer's instructions. For the EndoZyme® test kit, this instruction included the use of spike controls alongside all tested polysaccharides. Linear regression models were used for both kits to construct the calibration curves based on log concentration and log fluorescence or log absorption readings at both labs. For the EndoZyme test kit, LPS standards in a range from 0.005 to 5 EU/ml were used. In comparison, the ToxinSensor™ test kit can be used either in the range of 0.01–0.1 or in the range of 0.1–1 EU/ml. At both labs, spiking was performed by adding LPS to

a final concentration of 0.5 EU/ml to appropriate dilutions of the polysaccharides. Dilutions were chosen to achieve a measured endotoxin concentration of approximately 0.5 EU/ml in the non-spiked sample in order to ensure a spike value of similar magnitude as the measured endotoxin concentration of the diluted samples.

2.4.2. LPS quantification with HEK-Blue hTLR4 assay

HEK-Blue hTLR4 cells are transgenic for the cell surface expressed hTLR4, MD-2 and CD14 receptors and contain a downstream reporter system resulting in secretion of secreted embryonic alkaline phosphatase (SEAP) under the control of NFκB and AP1 promoters. HEK-Blue hTLR4 cells were detached using a cell scraper when 90–95% confluence was reached and 0.5×10^6 cells were transferred to each well of a 96-well Poly-D-Lysine coated plate. After O/N incubation at 37 °C in an atmosphere containing 5% CO₂, we added 0.0001–100 EU/ml LPS (E. coli O11: B4, 1 EU=0.15 ng/ml, Sigma, St. Louis, MO, USA) or polysaccharides to a final concentration of 300 ng/ml or 2.5 mg/ml to the cells and the plate was incubated for 8 h (37 °C; 5% CO₂). TLR4 stimulation resulted in SEAP secretion, which was quantified by mixing 20 μl of supernatant (depleted from cells by centrifugation at 450g for 5 min) with 180 μl of Quanti-Blue™ in a new flat bottom 96-well plate. The plate was incubated for 3 h at 37 °C and absorption at 655 nm was determined every hour using a spectrophotometer (TECAN, Giessen, The Netherlands).

2.5. Alkaline-ethanol treatment for LPS inactivation

Before LPS inactivation, all labware was washed 3 times with 1.0 M (M) NaOH and rinsed with LPS-free MilliQ. As an initial trial, 6 mg of lentinan isolated from shiitake (isolated as described in Tomassen et al. (2011)) was dissolved in 1 ml of 0.1, 0.25, 0.5, 1 or 2 M NaOH. During the incubation at 56 °C (gently shaken) a sample of 250 μl was taken after 30, 150 and 300 min. Directly after sampling equal molar amounts of HCl were added for an approximate neutralisation. The samples were 4 times diluted with LPS-free water (Lifetechnologies-Gibco) and the remaining LPS concentration was measured using the ToxinSensor test kit. For the preparative experiment, 50 mg of all polysaccharides were mixed with 10 ml of 0.1 M or 0.5 M NaOH in 60% ethanol in a 50 ml tube. The polysaccharides were incubated for 5 h at 4 °C or 56 °C while mildly shaken. Next, 1 ml of 1.0 M or 5.0 M HCl was added for an approximate neutralization of the mixture. Finally, absolute ethanol was added to give a combined total volume of 40 ml and samples were stored O/N at 4 °C. Polysaccharides were recovered by centrifugation for 20 min at 3320g and washed three times with 40 ml 60% ethanol. Finally, polysaccharides were lyophilized.

2.6. Cell viability assay

Cell viability of HEK-Blue hTLR4 cells after stimulation was determined with the MTT test. Following polysaccharide stimulations, cells were washed and incubated with 10 μl medium containing 5.5 mg/ml MTT (Merck, Darmstadt, Germany) for 2 h at 37 °C and 5% CO₂. Next, 10 μl DMSO: ethanol (1:1) was added to each well and the plate was mildly shaken for 5 min. The absorbance measured at 570 nm using an ELISA plate reader (TECAN infinite® M200 pro NanoQuant) showed the conversion of MTT to formazan indicative of mitochondrial activity and cell viability.

2.7. Size exclusion chromatography with refractive index detection (SEC-RI)

Original and alkaline-ethanol treated samples were dissolved in eluent (50 mM Na₂SO₄) at approximately 1.5 mg/ml. The HPLC

system consisted of a pump (Dionex P680), a Spectaphysics AS3500 auto injector, a guard-column (Tosoh PWXL), two serially connected columns (Tosoh TSK-gel G6000 PWXL followed by G5000 PWXL), maintained at 40 °C and a refractive index detector (Shimadzu RID-6A). The eluent was delivered at a flow rate of 0.5 ml/min. Injection volume was 95 μl and raw data was collected by Chromeleon software v. 6.8 (Dionex). All data was processed by PSS WINGPC Unichrome software (Polymer standard service, Mainz, Germany) and analysed by peak position calibration relative to pullulan standards fitted with a proprietary third order polynomial regression algorithm (PSS 3).

3. Results

3.1. LPS quantification in polysaccharide preparations with commercial LAL-based test kits

The LPS content of the different polysaccharide samples was measured in two different labs with two different LAL-based test kits, Endozyme® and ToxinSensor™. Analysis of LPS standards resulted in linear calibration curves with coefficients of determination above 0.968 for all Endotoxin and 0.952 for all ToxinSensor measurement series (Supplementary fig. 1). As described in the methods section, the two test kits strongly differ in the ranges of detection. The Endozyme kit detection range is from 0.005 EU/ml to 5 EU/ml and the ToxinSensor detection range is from 0.01 to 0.1 or 0.1–1 EU/ml. The back calculated concentrations of the different standard solutions are shown in Table 1 for all measurement series. Except for one out of 20 data points, all back calculated standard concentrations for the Endozyme kit were within the acceptable range stated by the manufacturer (60–140%; Table 1). Although the manufacturer of the ToxinSensor kit does not provide a range, results demonstrated a similar back calculated standard range as the Endozyme kit with one outlier (54%) out of 12 data points outside the 60–140% range. Despite these standardised calibration curves the absolute values of determined LPS contents in the different polysaccharide preparations generally deviated between the two test kits and the two labs, although trends were similar. The largest difference observed between labs for the Endozyme test kit was around 7-fold, e.g. lab 1 and 2 detected 115 and 756 EU/mg for the RG-1 sample, respectively (Fig. 1A and B). The smallest difference in detection was 2.5-fold, e.g. lab 1 and 2 detected 0.05 EU/mg and 0.02 EU/mg, respectively, for the Wellmune Soluble sample. For the ToxinSensor kit trends were also similar between labs but the highest difference in detection was 33-fold, e.g. lab 1 and 2 detected 525 and 16 EU/mg, respectively, in the Naxus sample (Fig. 1C and D). The smallest difference in detection was again observed for the Wellmune Soluble sample and was about 1.3-fold, e.g. lab 1 and 2 detected 0.05 EU/mg and 0.04 EU/mg, respectively. Observed differences between test kits were largest for Naxus (17-fold; Endozyme: 31 versus ToxinSensor: 525 EU/mg) and OBW90 (157-fold; Endozyme: 0.05 versus ToxinSensor: 7.93 EU/mg) in lab 1 (Fig. 1A and C). In lab 2, Naxus (7-fold; Endozyme: 104 versus ToxinSensor: 16 EU/mg) and OBW90 (142-fold; Endozyme: 0.02 versus ToxinSensor: 2.84 EU/mg) demonstrated the largest variation in LPS detection between the two test kits (Fig. 1B and D).

For the EndoZyme® test kit, the manufacturer instruction requires the inclusion of spike controls alongside all tested samples. Measured LPS values of samples are only deemed valid if the recovery of the corresponding spike controls is between 50% and 200%. In lab 1, LPS measurements in Naxus and RG-1 could not be validated because the recovery of the corresponding spike controls was 30% and –59%, respectively (Fig. 1E). For lab 2 the averages of spike control recoveries were all between 50% and 200% (Fig. 1F).

Table 1
Endotoxin test kit performance evaluation by assessing internal LPS standards.

Endozyme ^a						ToxinSensor ^b			
Nominal standard concentrations [EU/ml] ^d	Back calculated standard concentrations ^c					Nominal standard concentrations [EU/ml] ^e	Back calculated standard concentrations ^c		
	Test 1	Test 2	Test 3	Test 4	Test 5		Test 1	Test 2	Test 3
5	104	102	89	109	139	1	96		
0.5	78	95	123	98	75	0.5	120		
0.05	104	102	93	81	65	0.25	95		
0.005	84	n.a	98	116	146	0.1	54	97	96
						0.05		117	120
						0.025		90	86
						0.01		78	77

n.a.=not applicable

^a Test 1 and 2 were performed by lab 1 and test 3–5 by lab 2.

^b Test 1 was performed by lab 1 and test 2 and 3 by lab 2.

^c Back calculations of standard concentrations were performed using the standard curves presented in supplementary Fig. 1 and shown as percentage of nominal.

^d Concentrations provided by Endozyme manufacturer to construct the standard curve.

^e Concentrations provided by ToxinSensor manufacturer to construct the standard curve.

However, the high standard deviation of the spike recovery values for RG-1 indicates problems with LPS determination in the RG-1 sample also in lab 2. These results demonstrate difficulties to obtain reliable absolute values for LPS contents in different polysaccharide samples with two commercial LAL based test kits.

3.2. LPS detection in polysaccharide preparations based on HEK-Blue hTLR4 reporter cells

As an alternative method to detect LPS in polysaccharides we used HEK-Blue hTLR4 cells that are transfected with the LPS receptor complex. LPS-mediated TLR4/MD2/CD14 activation was quantified by increased absorbance following SEAP activity. To verify the sensitivity of the HEK-Blue hTLR4 cells and quantify LPS contamination in polysaccharide preparations using the HEK-Blue hTLR4 cells, we stimulated the cells with different LPS concentrations (Fig. 2A). Using a 5-parameter logistic curve fitting to convert SEAP reporter activity into EU/ml this revealed a detection range between 0.011 and 1.176 EU/ml for the HEK-Blue hTLR4 cells. Stimulation of HEK-Blue hTLR4 cells with a number of polysaccharides at 2.5 mg/ml visually appeared to result in reduced cell viability, which was confirmed with an MTT assay (Fig. 2B). Surprisingly, OBW90 also reduced cell viability when using a relative low concentration (i.e., 300 ng/ml), in contrast to the other polysaccharides (data not shown). Measurements with low polysaccharide concentrations resulted in significant HEK-Blue hTLR4 responses and therefore indicated LPS contaminations in LCES (2600 EU/mg), Naxus (53 EU/mg) and RG-1 (121 EU/mg) (Fig. 2C). In contrast, responses to Wellmune Soluble and OBW90 samples were below detection levels of 0.011 EU/ml, which, due to the low polysaccharide concentrations, only indicates that LPS contaminations are below 38EU/mg. Surprisingly, detected LPS contaminations were only 2.3-fold (LCES), 1.2-fold (Naxus) and 3.5-fold (RG-1) different between the average Endozyme kit results (Fig. 1A and B) and HEK-Blue hTLR4 cells (Fig. 2C). In addition, the lower detection limit of the HEK-Blue hTLR4 assay (i.e., 0.011 EU/ml) is also similar to the Endozyme kit (i.e., 0.005 EU/ml).

3.3. Optimal conditions for alkaline-ethanol-based LPS inactivation

To allow analysis of *in vitro* and *ex vivo* immune-modulatory potential of the polysaccharides we needed to remove LPS activity. To this end we adapted a method based on alkaline-ethanol treatment. In a pilot experiment a lentinan extract (Tomassen et al., 2011) was treated at 56 °C with different alkaline

concentrations and for various incubation times to determine conditions for maximal LPS inactivation. LPS contents after treatments were initially measured using the ToxinSensor test kit. The relative LPS content was lowest after 2.5 h with 0.5 M NaOH or 5 h with 0.1 M NaOH (Supplementary fig. 2). For the main experiment, the NaOH concentration of 0.1 M in combination with an incubation time of 5 h was selected and we included an additional temperature setting at 4 °C. We selected these conditions because a lower alkaline concentration and a lower treatment temperature are assumed to lead to less degradation of the polysaccharides.

3.4. Alkaline-ethanol treatment of polysaccharide preparations

In the main experiment, the above selected alkali treatments (0.1 M NaOH in 60% ethanol at 4 °C and 56 °C) were applied to all samples. After treatment, the relative yield of the treated samples was determined by weighing the lyophilized samples (Supplementary fig. 3A). This resulted in yields between 60% and 110%. In general, RG-1 and Wellmune Soluble showed the lowest yields which might be related to incomplete recovery during ethanol precipitation. The efficient removal of neutralized alkali was confirmed by osmolality measurements (Supplementary fig. 3B). This shows that the lyophilized samples did not contain any significant amounts of salt, which is important both with respect to calculated yields and because increased osmolality could affect cell viability in subsequent cell culture experiments.

3.5. Effect of alkali-ethanol treatment on LPS contaminations in polysaccharide preparations

LPS contamination of the alkaline-ethanol treated polysaccharide preparations was estimated with the EndoZyme test kit. The LPS contamination detected in the treated polysaccharides at both temperatures was lower compared to the untreated polysaccharides (Fig. 3A). Treatment at 4 °C resulted in detectable residual LPS in the LCES and Naxus samples, whereas treatments at 56 °C reduced LPS contaminations below the detection limit (0.0017 EU/mg) for all polysaccharides. However, the corresponding spike controls of the treated polysaccharides were outside the acceptable range for most of the samples (Fig. 3B). Only Wellmune Soluble treated at 4 °C and 56 °C and LCES and Naxus treated at 4 °C demonstrated spike recovery between 50% and 200%. Therefore, the measured LPS contents are strictly only valid for these samples. In order to validate endotoxin concentrations in all preparations we employed the HEK-Blue hTLR4 reporter cell

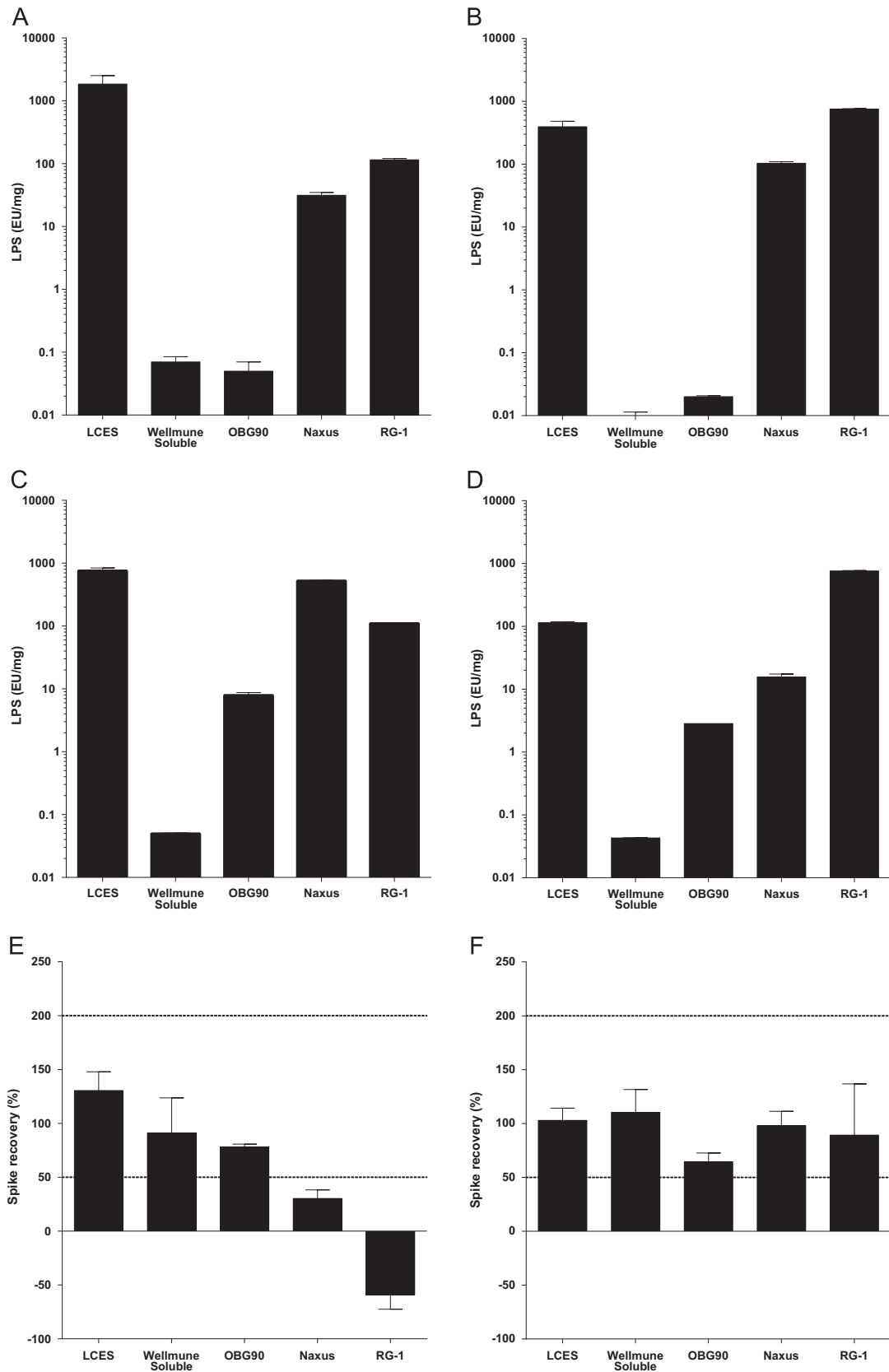


Fig. 1. LPS content and spike recovery in polysaccharide preparations as determined by LAL-based test kits. LPS contamination in LCES, Wellmune Soluble, OBG90, Naxus and RG-1 was determined using the EndoZyme test kit (A, B) or the ToxinSensor test kit (C, D) at lab 1 (A, C) and lab 2 (B, D). Spike recovery (percentage of detected spike) in the polysaccharide preparations was determined with the EndoZyme test kit at lab 1 (E) and lab 2 (F). The dotted lines represent the threshold level of a valid spike recovery. Bars represent averages \pm SEM of $n=2-6$ measurements.

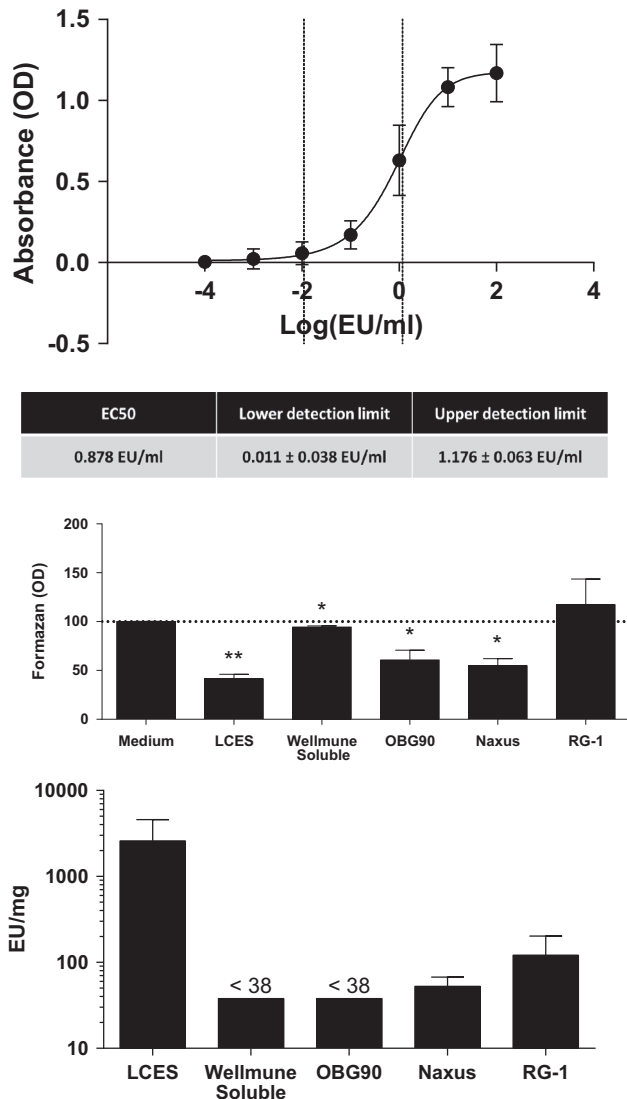


Fig. 2. HEK-Blue hTLR4 cells allow determination of functional LPS contamination in polysaccharides. HEK-Blue hTLR4 cells were incubated for 8 h with 0, 0.0001, 0.001, 0.01, 0.1, 1, 10, or 100 EU/ml LPS (A), or 2.5 mg/ml (B) or 300 ng/ml (C) untreated LCES, Wellmune Soluble, OBG90, Naxus and RG-1. In A alkaline phosphatase activity was determined and LPS concentrations of n=6 independent measurements are log(10) transformed and absorbance values plotted ± standard error using a 5-parameter logistic equation with best fit values for EC50, lower and upper detection limits depicted in EU/ml. The lower and upper detection limits are indicated in the graph with vertical dashed lines. In B MTT was added and conversion to formazan was detected by measuring absorbance at 570 nm and shown relative to medium (set at 100%). Bars represent averages+SEM of n=4 to 6 independent measurements. LPS contaminations in polysaccharides are depicted in C in EU/mg following analysis of alkaline phosphatase activity with the 5-parameter logistic equation and correction for the dilution factor. Bars represent averages+SEM of n=3 independent measurements. Statistically significant differences of polysaccharides to medium were calculated with two-tailed Student's *t*-tests: * = $p < 0.05$; ** = $p < 0.005$.

assay. Analysing LPS contaminations at 300 ng/ml resulted in no significant responses (data not shown), demonstrating the treatment was effective but also only indicating LPS contaminations were below 38 EU/mg. Surprisingly, analysing 2.5 mg/ml of treated LCES and Naxus did not, in contrast to untreated polysaccharides, result in reduced cell viability (Fig. 4A). HEK-Blue hTLR4 responses to 2.5 mg/ml stimulation of treated polysaccharides confirmed that LPS contaminations are reduced at both temperatures and most pronounced following treatment at 56 °C (Fig. 4B). In fact, this reduces LPS contamination in LCES and Naxus below detection

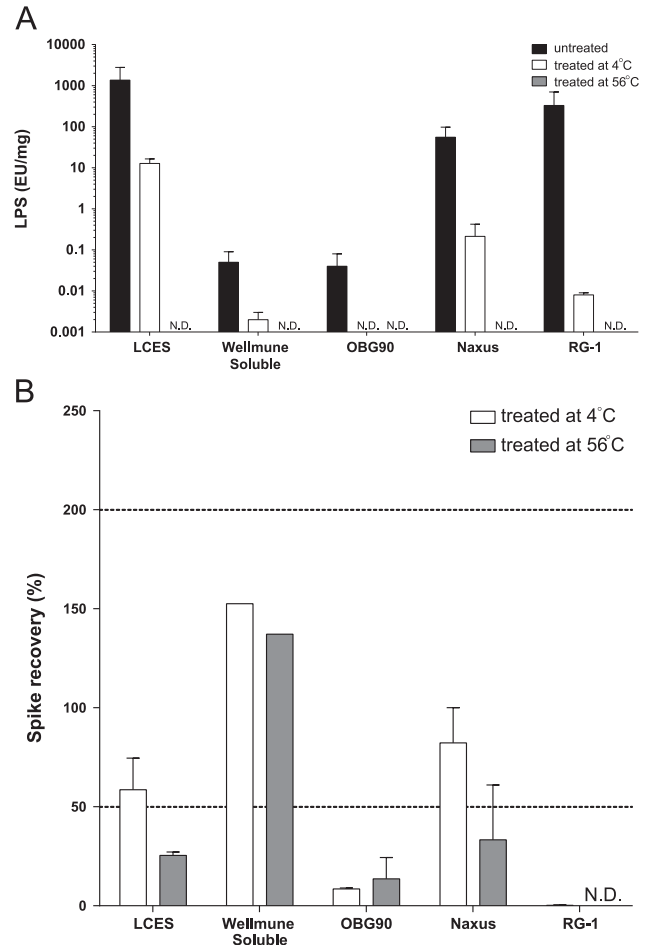


Fig. 3. Endozone measurements suggest efficient LPS inactivation by alkaline-ethanol treatment but spike recovery indicates results are unreliable. LPS contamination of untreated or at 4 °C and 56 °C alkaline-ethanol treated LCES, Wellmune Soluble, OBG90, Naxus and RG-1 was determined with the Endozone test kit (A) and spike recovery was determined in treated samples (B). Bars represent averages+SEM of n=1 or 2 independent measurement and N.D. is non-detectable.

limit (0.0046 EU/mg) and RG-1 (0.0143 EU/mg) below LPS contaminations observed for Wellmune Soluble. Indeed, the increased sensitivity resulting from testing higher concentrations of polysaccharides demonstrated low but detectable LPS contamination in Wellmune Soluble (*i.e.*, 0.177 EU/mg). For OBG90 the LPS contamination was also below threshold levels, but measurements were unreliable due to the reduced cell viability.

3.6. Effect of alkali-ethanol treatment on molar mass distributions of polysaccharide preparations

To investigate whether the polysaccharides are markedly depolymerized during treatment, we measured the molar mass distributions (MMD) of the treated and untreated polysaccharides using SEC-RI. For the unimodal distributions of OBG90 and Naxus we reported the weight average MW (M_w) relative to pullulan standards (Table 2). OBG90 alkaline-ethanol treatment at 4 °C did not lead to any degradation, while treatment at 56 °C resulted in a profound degradation with a high variation between treatment replicates (Fig. 5A and Table 2). For Naxus there was no difference between treatment at 4 °C and at 56 °C. Both led to a slight broadening of the molar mass distribution and a slight increase of the calculated M_w (Fig. 5B and Table 2).

For a quantitative comparison of the polysaccharides with a polymodal distribution we divided the distributions into two

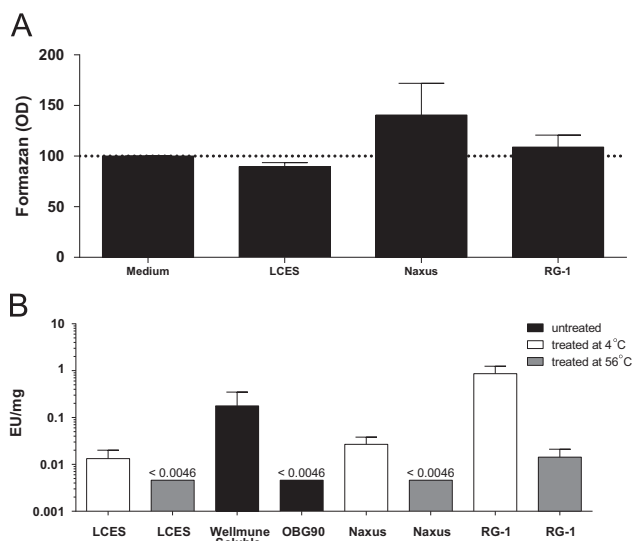


Fig. 4. HEK-Blue hTLR4 show that alkaline-ethanol efficiently inactivated LPS in polysaccharide preparations. HEK-Blue hTLR4 cells were incubated for 8 h with 2.5 mg/ml of untreated Wellmune Soluble and OBW90 and at 56 °C alkaline-ethanol treated LCES, Naxus, and RG-1. In A we detected formazan following incubation with treated LCES, Naxus and RG-1 as described for Fig. 2B. Bars represent averages + SEM of n=4–6 independent measurements. No statistically significant differences of polysaccharides to medium were found as calculated with two-tailed Student's *t*-tests. In B we detected alkaline phosphatase activity as described for Fig. 2C. Bars represent averages+SEM of n=2 to 5 independent measurements.

different peak areas and calculated the M_w for both peaks as well as the corresponding contribution of each peak to the total area (Table 2). The polymodal molar mass distribution of RG-1 can be roughly divided into one peak with M_w of around 10,000 g/mol (peak A), constituting 55% of the polysaccharide, and one peak with M_w around 100,000 g/mol (peak B), constituting about 45% of the polysaccharide (Fig. 5C and Table 2). Alkaline-ethanol treatment at 4 °C did not change the M_w of the two peaks, but resulted in a change in their proportion. There was a large variation in the effect of treatment at 56 °C on RG-1, which resulted in a profound change of the B peak in one of the parallels, while the other more closely resembled the MMD of the untreated sample. In the untreated LCES the B peak showed a M_w of 12,000,000 g/mol and

constituted about 74% of the sample (Fig. 5D and Table 2). The A peak was defined as the broad remainder of the distribution with a M_w of 370,000 g/mol and constituted 26% of the sample. Alkaline-ethanol treatment of LCES at 4 °C or 56 °C resulted in a dramatic decrease of the area of the B peak to 20% and 11%, respectively, of the total area even though the M_w of the B peak remained above 10,000,000 g/mol for both treatment temperatures (Table 2). The A peak correspondingly increased in proportion, with a slight reduction in M_w . Alkaline-ethanol treatment of Wellmune Soluble at 4 °C did not affect the MMD of the polysaccharide except for a slight decrease in M_w of the B peak (from 86,000 to 53,000 g/mol; Fig. 5E and Table 2). Treatment at 56 °C, on the other hand, resulted in a change of the MMD distribution into a broad but essentially unimodal distribution.

4. Discussion

To detect *in vitro* polysaccharide-mediated immune-modulation it is crucial to control endotoxin contaminations. Here we describe the quantification of LPS in different polysaccharide preparations with 2 commercial kits and a cellular system. In both kits, LPS detection is depending on Lipid A-mediated activation of complement factor C and the following LAL cascade. According to the back calculated standards, both kits performed within specification. The LPS content of an external LPS control (E.coli O11: B4, Sigma) was accurately quantified with both test kits (data not shown). Additionally, the Endozyme test kit has given reliable values and valid spike controls for a range of protein samples tested in both labs (data not shown). However, the lack of valid spike controls for some of the polysaccharide samples (especially the alkaline-ethanol treated polysaccharides; Fig. 3B) and varying results between the ToxinSensor and Endozyme kits demonstrated that these kits cannot be universally used for all polysaccharide preparations. This is in agreement with the experience of a specialist ultrapure biopolymer producer that found specific LPS quantification kits must be selected for each different type of polysaccharide (Personal Communication, Novamatrix, FMC Health and Nutrition, Sandvika, Norway). We have previously shown that a cereal β -glucan inhibited the binding of LPS to polymyxin (Rieder et al., 2013), probably due to steric hindrances.

Table 2
Molar mass distributions of untreated and alkaline-ethanol treated polysaccharides preparations.

Sample	Distribution ^a	Parameter ^b	Untreated ^c	4 °C ^d	56 °C ^d
OBW90	unimodal	M_w (g/mol)	1,440,000	1,380,000 ± 13,000	402,000 ± 305,000
		area (%)	100	100	100
Naxus	unimodal	M_w (g/mol)	43,000	67,500 ± 10,000	63,000 ± 2200
		area (%)	100	100	100
LCES	Peak A	M_w (g/mol)	373,050	248,000 ± 8000	196,000 ± 18,000
		area (%)	26.3	80.2 ± 1.8	89.1 ± 1.8
	Peak B	M_w (g/mol)	12,670,000	11,000,000 ± 370,000	10,270,000 ± 180,000
		area (%)	73.7	19.8 ± 1.8	10.9 ± 1.8
RG-1	Peak A	M_w (g/mol)	8900	10,200 ± 90	9800 ± 80
		area (%)	55.1	66.3 ± 6.5	59.6 ± 9.6
	Peak B	M_w (g/mol)	114,000	105,000 ± 12,000	133,000 ± 41,000
		area (%)	44.9	33.7 ± 6.5	40.4 ± 9.6
Wellmune Soluble	Peak A	M_w (g/mol)	6670	7040 ± 1	7370 ± 70
		area (%)	36.5	32.8 ± 1.3	41.3 ± 1.4
	Peak B	M_w (g/mol)	86,200	53,200 ± 270	44,000 ± 850
		area (%)	63.5	67.2 ± 1.3	58.7 ± 1.4

^a Unimodal distributions show only one peak. To facilitate a clear presentation of the observed trends, all polymodal distributions were divided into two peak areas only (Peak A and B).

^b The shown parameters include the weight average molecular mass (M_w) of the different peak areas calculated relative to pulullan standards and their relative contribution to the total area in %.

^c Untreated polysaccharide preparations were measured once.

^d The treatment of polysaccharide preparations with alkaline-ethanol at 4 °C or 56 °C was performed twice and both replicate samples were analysed.

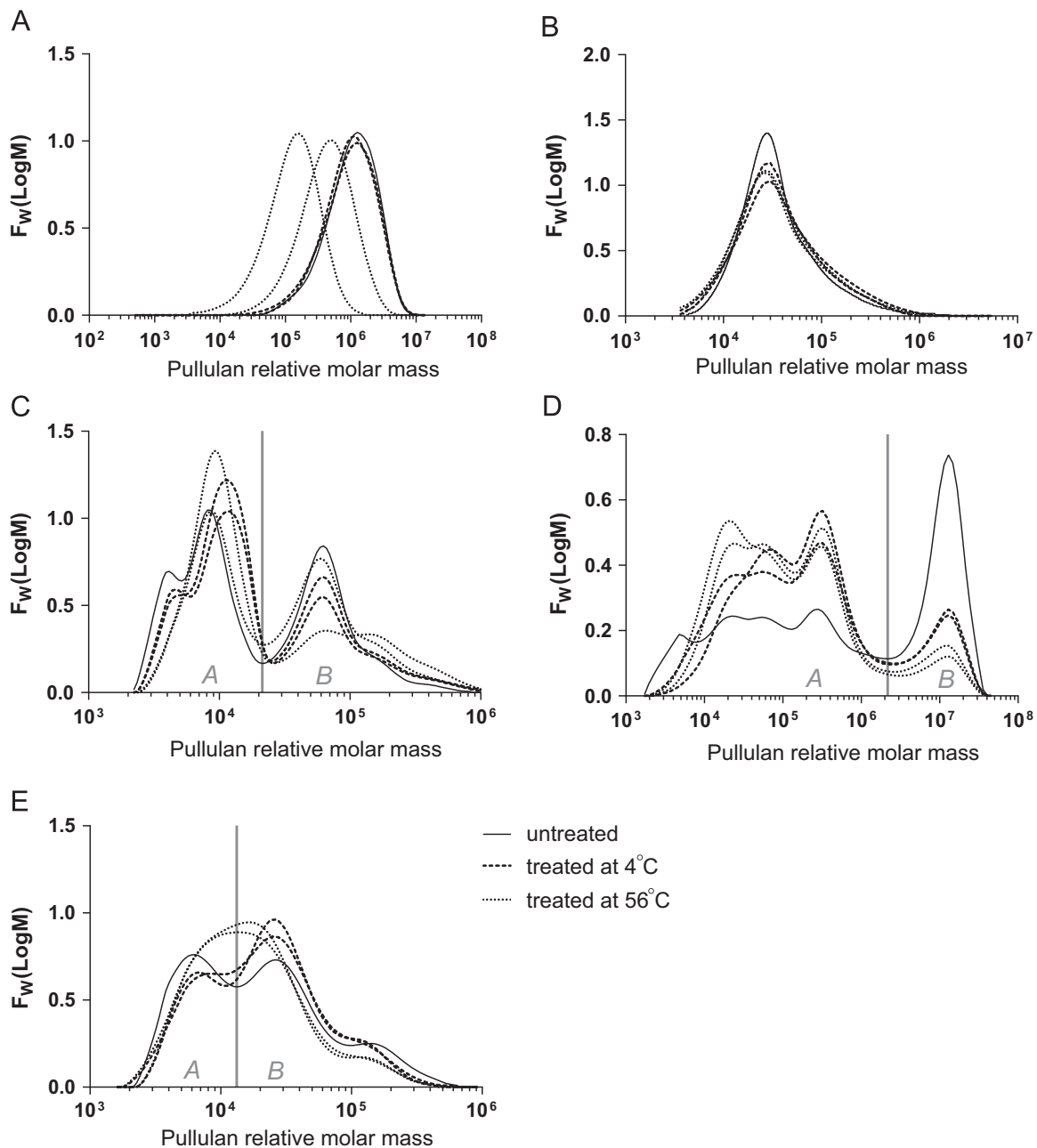


Fig. 5. Molar mass distributions demonstrate effects of alkali-ethanol treatment on polysaccharide preparations. Molar mass distributions of OBW90 (A), Naxus (B), RG-1 (C), LCES (D) and Wellmune Soluble (E) were determined before (solid line) and after alkaline-ethanol treatment at 4 °C (dotted line) and 56 °C (dashed line). The figure shows weight-differential molar mass distributions in logarithmic scale ($F_w(\log M)$) relative to pullulan. Alkaline-ethanol treatment at 4 °C and 56 °C of each polysaccharide preparation was performed twice and both replicate samples are presented.

It is therefore possible that polysaccharide LPS interactions influence the LPS activity/availability in LAL assays.

To circumvent some of the problems with the LAL-based test kits we adapted an alternative method to detect LPS in polysaccharides. The employed HEK-Blue hTLR4 reporter cells are transgenic for the cell surface expressed hTLR4, MD-2 and CD14 receptors and contain a downstream reporter system resulting in secretion of secreted embryonic alkaline phosphatase (SEAP) under the control of NF κ B and AP1 promoters. A major advantage of using this cell assay is that its method of LPS detection is representative for other cellular (immune) assays. Although both LAL-based kits and CD14/MD2/TLR4-mediated cellular recognition are based on detection of lipid A, only cellular recognition requires lipid A to adopt a certain aggregate conformation (Gutsmann et al., 2010) which can lead to

discrepancies in LPS detection between both methods. Surprisingly, however, detected LPS contaminations and lower detection thresholds were similar between the Endozyme test kit and the HEK-Blue hTLR4 cells. The detection limit of the HEK-Blue hTLR4 assay is also below lower limits of LPS responses observed by various immune cell types (~ 0.2 EU/ml (Schwarz et al., 2014)). The HEK-Blue hTLR4 assays could lead to false-positive results when polysaccharides have potential to interact with the TLR-4 receptor as has been demonstrated in multiple studies (Capitan-Canadas et al., 2014; Chen et al., 2009; Lehmann et al., 2015; Li et al., 2016; Ortega-Gonzalez et al., 2014; Sahasrabudhe, Dokter-Fokkens, & de Vos, 2016; Tsuji et al., 2015; Vancraeynest et al., 2016; Vogt et al., 2013; Zhu et al., 2014). However, none of those studies verify the absence of LPS contaminations below 0.2 EU/ml, leaving the distinct possibility the

observed TLR-4 stimulating effects in fact result from LPS activity. Another potential limitation of the cellular assay is that compounds might reduce cell viability leading to false-negative LPS contamination. Initial testing of untreated samples therefore requires titrations of the test compound to determine cell viability and prevent false negative alkaline phosphatase responses. It appeared that HEK-Blue hTLR4 cells are sensitive to high concentrations of polysaccharides with high LPS contaminations (Fig. 2B) since similar polysaccharide concentrations following alkaline-ethanol treatment at 56 °C did not affect cell viability (Fig. 4A). This notion was substantiated by the fact that untreated Wellmune Soluble, containing <0.2 EU/mg LPS, only induced limited reduction in cell viability (94%). In contrast, untreated OBW90 also has a low LPS contamination as determined with the Endozyme kit and spike control in both labs (0.035 EU/mg; Fig. 1), but did reduce cell viability (Fig. 2B). When stimulating immune cells (*i.e.*, primary macrophages) with OBW90 we observed a significantly lowered mRNA yield, indeed also suggesting lowered cell viability and/or increased cell death. However, follow-up analysis using flow cytometry and Annexin VI and PI stainings indicating reduced cell viability and cell death, respectively, did not show differences between medium and OBW90 stimulated cells (Tang et al., manuscript in preparation) leaving the cause of the reduced mRNA yield and MTT values in HEK-Blue hTLR4 cells unclear.

Analysing LPS contaminations in HEK-Blue hTLR4 cells at low polysaccharide concentrations indicated that LCES, Nexus and RG-1 contained significant amounts of LPS and therefore required treatment. We performed the alkaline-ethanol treatment because alkali conditions are known to induce de-O-acylation and therefore will release phosphate-ester groups and ester-bound fatty acids in Lipid A (Rosner, Tang, Barzilay, & Khorana, 1979). It has been shown that the phosphate groups and fatty acids are critical for Lipid A bioactivity (Bentala et al., 2002; Fukuoka et al., 2001) and LAL-based detection (Gutsmann et al., 2010). Water-based alkali treatment to degrade lipid A moieties have been described as early as the 1980's. In fact, alkaline treatment of both LPS and contaminated polysaccharide samples has been used as a detoxification strategy (Adam, Vercellone, Paul, Monsan, & Puzo, 1995; Mikami, Nagase, Matsumoto, Suzuki, & Suzuki, 1982). Alternatively to de-O-acylation of the Lipid A moiety of LPS, the treatment may have interfered with physical interactions between LPS and the polysaccharides. Under alkali conditions, polysaccharides will be present in a more open, elongated form due to interchain repulsion with also a consequent disruption of aggregates (Podzimek, 2011). Such conformational changes may have allowed LPS or the residual Lipid A to be washed away. Conformation changes like the disruption of aggregates may also have resulted in reduced activity since it has been suggested that the biological activity of LPS may depend on the presence of aggregated LPS units (Mueller et al., 2004). In a previous study, the stability of the carrageenan backbone during acid desulphation was improved in the gel-state compared to carrageenan in aqueous solution (Zhang, Piculell, Nilsson, & Knutsen, 1994). The alkaline-ethanol treatment was therefore carried out in 60% ethanol with the polysaccharides suspended, but not solubilized. This approach has the additional advantage of simplifying polysaccharide recovery and salt removal after neutralization. Alkaline-ethanol treatment of LCES, Nexus and RG-1 preparations at 56 °C consistently resulted in a dramatic reduction in residual LPS contamination to <0.02 EU/mg (Fig. 4B) which is below immune cell response thresholds (Schwarz et al., 2014). Untreated Wellmune Soluble already demonstrated a LPS contamination sufficiently low to allow *in vitro* immune analysis (0.177 EU/mg). OBW90, like LCES and Nexus treated at 56 °C, contained LPS contaminations below detection threshold, but also showed strongly reduced cell viability which might prevent efficient SEAP responses to LPS levels and results in a false negative response. However, in case of OBW90 the spike controls validate

the Endozyme measurements showing a LPS contamination of 0.035 EU/ml (Fig. 1). Interestingly, the alkaline-ethanol treatment also reduced responses when stimulating HEK-hTLR2 cells with LCES or Nexus (data not shown). LTA is a classical ligand for TLR2 and contains D-alanine ester residues (Neuhaus, Linzer, & Reusch, 1974). Possibly, in parallel to LPS, de-esterification results in the inactivation of LTA, causing a reduction in TLR2 responses.

Alkaline-ethanol treatment may not only influence LPS content, but can also potentially alter the polysaccharide structure and lead to a degradation. We used SEC-RI as a screening tool to investigate changes in molar mass (Fig. 5). Especially for OBW90, which is a pure linear polysaccharide with a unimodal MMD, SEC-RI can be used to monitor random depolymerisation. Our results clearly demonstrate that alkaline-ethanol treatment at 56 °C leads to a profound degradation of OBW90, while treatment at 4 °C does not affect the MMD. For Nexus we observed a broadening of the unimodal MMD after alkaline-ethanol treatment at both temperatures. This may be related to changes in polysaccharide composition as Nexus is not only composed of arabinoxylan, but also contains a small defined fraction (5% of sample weight) of galactose containing polymers. We know from previous results that these galactose constituents can be removed from Nexus by a similar type of alkali treatment as employed in this study (data not shown). For RG-1 and Wellmune Soluble we observed minor differences between MMDs of untreated and alkaline-ethanol treated polysaccharides. For LCES we show that the B peak has a very high M_w of 12,000,000 g/mol which might represent aggregations. To calculate this relative M_w value, the pulullan calibration curve had to be extrapolated. The given value is therefore most likely an over-estimation. The obtained MMD can, nevertheless, be used to examine the effect of alkali treatment on LCES and we demonstrated a dramatic reduction of the contribution of the B peak to the total area from 74% in untreated LCES to 20% (4 °C) and 11% (56 °C) in the alkaline-ethanol treated LCES. The B peak of LCES may represent higher order structures like triple helices and re-natured triple helices. Fungal β -glucans in aqueous solution have been shown to adopt triple helix structures which appear important for some biological activity (Bohn & BeMiller, 1995; Falch, Espevik, Ryan, & Stokke, 2000; Zhang, Li, Xu, & Zeng, 2005). Alkali treatment has been shown to disrupt triple helical structures of fungal β -glucans when applying higher concentrations as used in this study (*e.g.*, 0.35 M). This denaturation was, however, at least partly reversible upon neutralisation followed by thermal annealing (Falch et al., 2000; Stokke, Elgsaeter, Brant, & Kitamura, 1991). Taken together, SEC-RI is a useful tool to visualize the effect of different alkaline-ethanol treatment conditions on the integrity of polysaccharide preparation. However, especially for complex polymodal and branched polysaccharide preparations, additional structural analysis like ¹HNMR or methylation analysis are required to fully investigate structural changes.

We have shown that alkaline-ethanol treatment can efficiently inactivate LPS in a variety of polysaccharide preparations and should be performed at 56 °C for optimal efficiency. The treatment should be combined with assays to validate LPS removal/inactivation as well as assays on polysaccharide structure since samples may differ in their susceptibility to alkali treatment and the efficacy of the treatment for LPS inactivation. SEC-RI proved to be a useful tool to monitor possible degradation of polysaccharide samples due to alkaline-ethanol treatment. Most importantly, however, remains the initial and proper testing of LPS contaminations, to prevent false positive immuno-stimulatory results (Gertsch et al., 2011). For polysaccharides we have demonstrated that endotoxin test kits are not universally applicable. Proper validation of results by the inclusion of spike control samples is crucial. The quantification of functional LPS by a HEK-Blue-hTLR4 based assay provides a good alternative method, but should be accompanied by a cell viability assay. Despite the laborious process, proper LPS quantification and inactivation/removal of LPS if

present in functional amounts are absolutely essential. In addition to that, the presence of other contaminants with potential immune-modulating properties has to be taken into account (Dinarello, O'Connor, Lopreste, & Swift, 1984; Zidek, Kmonickova, Kostecka, & Jansa, 2013). Specific enzymatic degradation of the polysaccharides can be used to confirm that a measured activity is truly related to a polymeric carbohydrate structure (Rieder et al., 2013). Only by identifying true polysaccharide-mediated immune-modulating activities, *in vitro* analysis can contribute to developing polysaccharide-based functional foods to aid in, for instance, treating high cholesterol levels, cancer, IBD and increasing general health (Gunness & Gidley, 2010; Schwartz & Hadar, 2014).

Author disclosure statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bcdf.2016.09.001>.

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